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Erythrocyte size and ploidy determination in green toads (*Bufo viridis* complex) from Middle Asia

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The sizes of erythrocytes in blood smears of green toads (*Bufo viridis* complex) from diploid and tetraploid Middle Asian populations (Iran, Turkmenistan, Uzbekistan and Kyrgyzstan) were compared by measuring their projection areas. Significant differences in the average values of erythrocyte areas between diploid and tetraploid toads were found. In some populations the values are in the overlapping range between both levels of ploidy. Erythrocyte areas variability, in relation to age, body size and altitude of the habitat, are discussed in the light of literature data on green toads and other polyploid anurans. The staining of erythrocytes with the Feulgen reaction and the microdensitometrical determination of the DNA content are described and considered to be an unequivocal method which does not require to kill the animals.

INTRODUCTION

Middle Asia (for ecogeographical definition, see WALTER & BRECKLE, 1986: 233, 275) is inhabited by diploid and tetraploid green toads (*Bufo viridis* complex; for taxonomic survey, see: ROTH, 1986, KUZMIN et al., 1988; STÖCK, 1995, 1997). In order to name diploid and polyploid forms from Middle and Central Asia which are all quite closely related to *Bufo viridis*, we use the term "*Bufo viridis* complex". This excludes some species (e.g., *Bufo calamita* or *Bufo surdus*) belonging to INGER's (1972) "*Bufo viridis* group", which was defined without information about the existence of polyploid forms. Since the discovery of tetraploid forms (MAZIK et al., 1976), the ploidy determination in these toads was based first on cytogenetic, cytophotometric and electrophoretic investigations, and concerned therefore only a quite low number of animals from each locality (BACHMANN et al., 1978; PISANETS, 1978; TOKTOSUNOV, 1984; KRJUKOV et al., 1985; BORKIN et al., 1986*a-b*, 1995; ORLOVA & UTEŠEV, 1986; ROTH & RÁB, 1986, 1987; WHU & ZHAO, 1987; KUDRJAVCEV et al., 1988; KUZMIN et al., 1988; MEŽŽERIN & PISANETS, 1990, 1995*a-b*; PISANETS, 1991, 1992*a-b*). On the other hand, in addition or exclusively, external morphological characters (which are sometimes misleading: ROTH, 1986, STÖCK, 1995, 1997) or form and size of the clutch (a questionable character too: KUZMIN, 1995: 94; STÖCK, unpublished) were used when the laborious methods mentioned above had to be limited to a few specimens or to avoid killing animals (ATAEV, 1987; PISANETS, 1987, 1992*a-b*; KUZMIN et al., 1988; TOJMASTOV, 1989, MEŽŽERIN & PISANETS, 1990; MEYER, 1991).

The aim of the present study was to develop additional methods for ploidy determination of many animals and suitable in field examinations in order to improve our knowledge about diploid and tetraploid green toads. Two methods were explored in this respect: (1) the simple microscopic measurement of projection areas of erythrocytes; (2) the microdensitometric measurement of DNA amounts.

MATERIAL AND METHODS

SPECIMENS

Samples were taken from Middle Asian animals in four diploid ($n = 36$) and six tetraploid populations ($n = 98$; see tab. 1, fig. 1) in spring 1993, 1994 and 1995 and autumn 1993 (for comparison, 13 animals from Halle/Saale, Germany, were used; permission by District Presidium Halle, 18.01.95). In these populations other investigations, such as morphological, bioacoustical and karyological analyses, were made (STÖCK, 1995, 1997, 1998). The skin of the tip of the fourth digit of each toad was scratched with a scalpel. This intervention seems to be of a little damage for an animal: in terrarium we observed the normal feeding of a toad as soon as a few seconds after manipulation and the wound was closed in one or two days. A small blood dose was used to make a blood smear on a clean microscopic slide (a single sample from each toad); in method (2) below, a "control smear" from an animal of known ploidy was made on the other end of the slide. The blood smears must not be polluted with skin mucus.

METHODS

Choice of methods

(1) In the past, the measurement of the size of cell nuclei took a leading part in the determination of polyploid individuals and species in Amphibia (reviews in: WEIß, 1954; HERTWIG et al., 1958). Moreover, relatively early, the empirically known ratio of genome size (DNA amount) to cell size was used for identification of polyploid amphibians, and erythrocytes were favoured because of their simple shape (reviews: GÜNTHER, 1977; AUSTIN & BOGART, 1982). Until today, some comparative studies (OLMO & MORESCALCHI, 1975, 1978; KURAMOTO, 1981; HORNER & MACGREGOR, 1983) verified that, both in Urodela and in Anura, there is a direct linear correlation between the DNA content and the nuclear volume, cell volume and cell surface of erythrocytes. BACHMANN et al. (1978) and KUDRJAVCEV et al. (1988) referred to greater cells of polyploid Middle Asian green toads, but with no statistical demonstration. HERRMANN (1989) suggested a micromorphological discrimination of diploid and tetraploid green toads using Raster Electron Microscope for distinguishing surface structures of the skin, but this author did not exactly determine the ploidy of his specimens.

Tab. 1. – Populations, numbers (*n*) and localities of specimens of green toads (*Bufo viridis* group) investigated.

Population Ploidy (diploid: 2 n = 22 tetraploid: 4 n = 44)	Abbreviation used in fig. 1	*n*	Localities of the populations, altitudes, dates of investigations
Bami 2 n = 22	a	23	38°37'N 56°38'E, Turkmenistan, Kysyl-Arvatskii Rayon, Kopet-Dag Mountains, valley approx. 25 km SE the station, S of the pass, alt. 750 m, March 1994
Bacharden 2 n = 22	b	3	38°14'N 57°31'E, Turkmenistan, Ashchabadskaya Oblast', approx. 10 km W Kelyata, alt. 500 m, April 1994
NE Iran 2 n = 22	c	9	37°38'N 55°29'E, frontier district near Turkmenistan, approx. 50 km NE Gonbad-e-Kāvūs, alt. 250 m, April 1994
Bishkek 2 n = 22	k	1	42°53N, 74°46'E, Kyrgyzstan, Bishkek, Botanical Garden, September 1993
Danata 4 n = 44	d	17	39°06'N 55°06'E, Turkmenistan, Ashchabadskaya Oblast', stream 2-4 km SE and warm spring approx. 4 km SE, alt. 200 m, April 1994
Bol'shoi Balkhan 4 n = 44 (and 2 n = 22?)	e	13	39°43'N 54°29'E, Turkmenistan, Nebit-Dagskii Rayon, Bol'shoi Balkhan Mountains, northern slope, approx. 15 km S Oglanly village, alt. 500 m, April 1994
Nuratau 4 n = 44	f	36	40°35'N 66°30'E, Uzbekistan, Dzhizakhskaya Oblast', Rayon Farish, Nuratau Nature Reserve, northern slope, alt 300-1600 m, May 1995
Tashkent 4 n = 44	g	6	41°16'N 69°13'E, Uzbekistan, alt. 450 m, April 1995
Chatkal 4 n = 44	h	5	41°35'N 70°07'E, Uzbekistan, 80 km E Tashkent, Chatkal Nature Reserve, 5 km E Burchmulla, alt. 900 m, April 1995
Issyk-Kul' 4 n = 44	i	21	42°29'N 76°20'E, Kyrgyzstan, northern bank near village Sary-Kamysh, alt 1670 m, April 1995

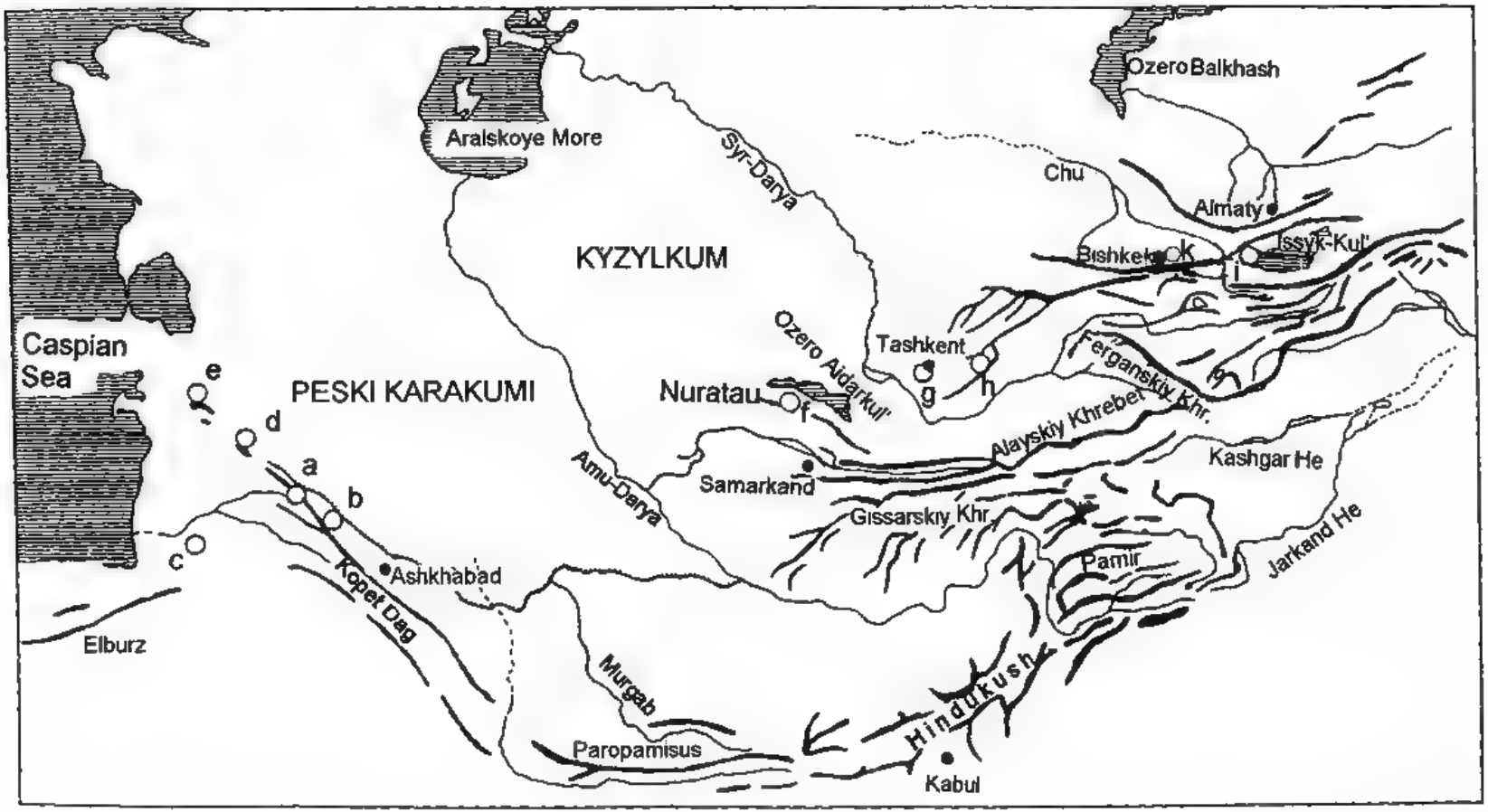

Fig. 1. Localities of the populations of green toads (*Bufo viridis* complex) investigated. Letters: see tab. 1. Map by Katrin SCHNEIDER & Matthias STÖCK, modified from WALTER & BRECKLE (1991: 275) For spelling of geographical terms, see ANONYMOUS (1993).

Tab. 2. - Average values of erythrocyte areas from populations investigated and of exemplary specimens and larvae which were karyologically investigated (counting of chromosomes). Numbers of toads involved in microdensitometry. For comparison a diploid population from central Europe (Halle/Saale) was added. *, values in the overlapping range between diploid and tetraploid populations.

Population (altitude)	Halle / Saale (110 m)	NE Iran (250 m)	Bacharden (500 m)	Bami (750 m)	Bishkek (850 m)	Bol'shoi Balkhan (500 m)	Danata (200 m)	Nuratau (300-1600 m)	Tashkent (430 m)	Chatka (1900 m)	Issyk-Kul' (1600 m)
Ploidy	2 n = 22	2 n = 22	2 n = 22	2 n = 22	2 n = 22	4 n = 44 (?)	4 n = 44	4 n = 44	4 n = 44	4 n = 44	4 n = 44
n of toads (blood smears)	13	9	3	23	1	13	17	36	6	5	21
Average erythrocyte projection areas (μm^2) - of populations	231 89	264.48	243.79	283 49	[279 78]	305.80*	340 12	334.81	323.56	333.56	374.73
- of toads karyologically investigated	-	270.49	-	283 64	279.78	292.49* 322.26	330 55 367 94	311 14* 317.28*	321 12	325.49	329.10 363 67
n of toads involved in microdensitometry	1	3	1	-	-	-	-	2	-	1	2
n of larvae karyologically investigated	2	-	2	2	-	-	2	3	2	-	-

Tab. 3. - LSD test. Significance level: $P < 0.05$. *, significant differences (shown in the lower triangle).

Population	Halle	NE Iran	Bacharden	Bishkek	Bami	B. Balkhan	Tashkent	Chatkal	Nuratau	Danata	Issyk Kul'
Halle											
NE Iran	*										
Bacharden	*										
Bishkek	*										
Bami	*	*									
B. Balkhan	*	*	*		*						
Tashkent	*	*	*	*	*						
Chatkal	*	*	*	*	*	*					
Nuratau	*	*	*	*	*	*					
Danata	*	*	*	*	*	*					
Issyk-Kul'	*	*	*	*	*	*	*	*	*	*	

(2) "The use of a scanning and integrating microdensitometer that is capable of making large numbers of accurate and reproducible readings from Feulgen-stained nuclei is probably the best way of determining the DNA content of cell nuclei" (HORNER & MACGREGOR, 1983), but time and condition of the main influencing steps of the Feulgen reaction (fixation and hydrolyses) "must be determined experimentally at the start of each and every programme of microdensitometry (...) for different tissues" (MACGREGOR & VARLEY, 1983: 233).

Description of methods

(1) Following GÜNTHER (1977) and POLLS PELAZ & GRAF (1988), the blood smears were only air dried. The microscopically visible projection areas of 30 or 50 randomly chosen, normally shaped erythrocytes were measured directly. We used the image analyses system CYDOK (Fa. Hilgers, see below) in combination with a transmission light microscope (ZETOPAN, Reichert, Vienna; 25 × enlarging objective) in the Hautklinik at the Martin-Luther-University. The minimum, maximum, average value and standard deviation of the areas were calculated. Photographs were taken using Orowopan 25 (15 DIN).

(2) Some air dried blood smears from field investigations which were stored more than one year and some few fresh blood smears (diploid: $n = 5$; tetraploid $n = 5$; tab. 2) from animals living in a terrarium were fixed according to HORNER & MACGREGOR (1983) for 10 min in fresh, ice cold methanol/glacial acetic acid (3/1). Slides were then processed through the Feulgen reaction using the method of SWIFT (1955). Hydrolyses for 18 min in 5 M HCl (Merck 1.09911) at 18°C, staining in Schiff's reagent (Merck 9034) for 90 min and washing in several changes of sulphite rinse (10 ml 1 N HCl + 10 ml 5 % solution of Natriummetabisulphite + 180 ml water) followed respectively HORNER & MACGREGOR (1983) and MINZUNO & MACGREGOR (1974). The stained preparations were dehydrated carefully through 95 % and 100 % ethanol and xylene and then mounted in a synthetic mountant and covered with coverglasses. One or two hundred nuclei were measured, in comparison with the samples of known ploidy used as a standard. We used the image analyses system CYDOK (Fa. Hilgers) and a transmission light microscope with a 40 × enlarging objective at a wavelength of 539 nm. The microscopic picture is transmitted life to a screen (enlarging factor approximately 1800 ×) via video-camera (effective size of the picture 420 × 420 pixels) The Feulgen dye content (the DNA amount) of a nucleus randomly chosen by the operator (mouse click) was determined by measuring the integrated optical density in the area covered by this nucleus.

RESULTS

ERYTHROCYTE AREAS

Erythrocyte areas in toads of both levels of ploidy

Erythrocytes are easily distinguishable from other blood cells by their ellipsoid shape. KHALIL & ELFEKY (1986) provided a detailed description of blood cells morphology in *Bufo viridis* from Egypt.

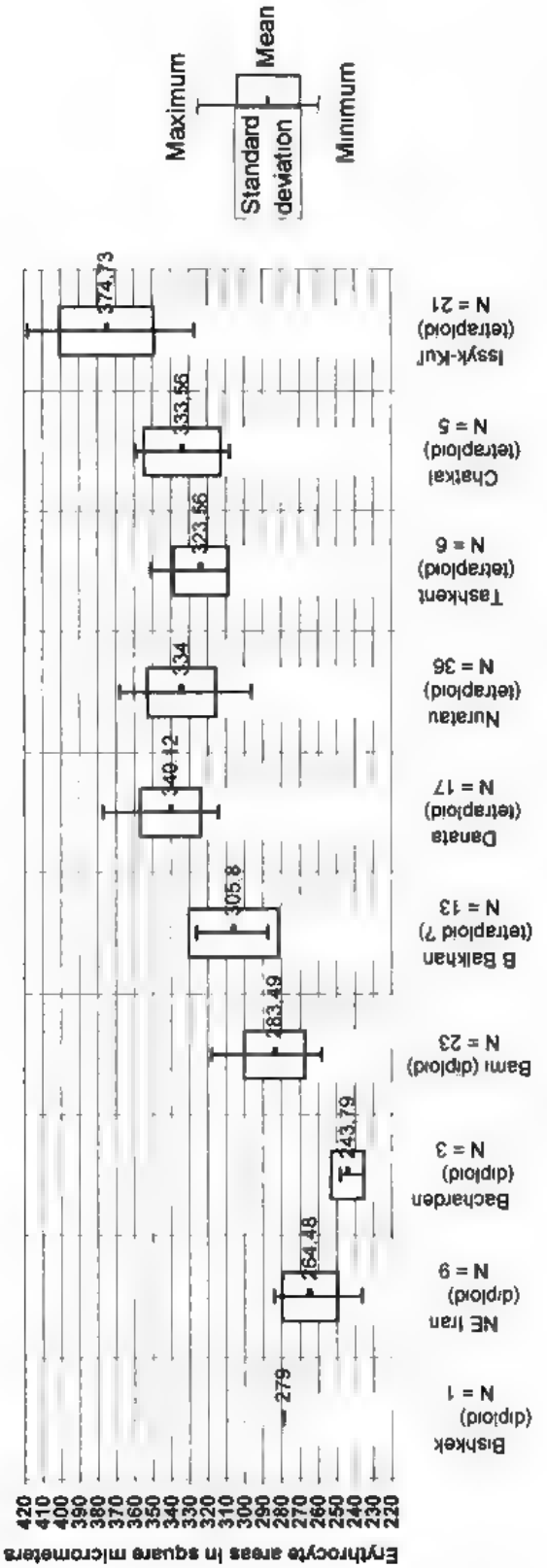

Fig. 2. – Minimum, maximum, standard deviation and average value (mean) of erythrocyte size in diploid and tetraploid green toad populations.

The average value of all diploid Middle Asian green toads investigated was 277.64 μm^2 (standard deviation, $s = 19.28$ μm^2), while in tetraploid toads it was 338.13 μm^2 ($s = 27.71$ μm^2). A Student t test for statistical comparison of the two means showed a significant difference with $P < 0.0001$. The variation range of all diploid individuals (235 μm^2 to 318 μm^2), whose number is also smaller, is distinctly smaller than that of tetraploid toads (287 μm^2 to 418 μm^2); this was especially caused by the values measured in the population Issyk-Kul' (see below).

Figure 2 displays the average value, the standard deviation and the variation range in each population. Average projection areas in the populations Danata, Nuratau, Tashkent and Issyk-Kul' in which exemplary specimens were identified as tetraploid were mostly larger than 310 μm^2. On the contrary, the diploid populations Bami, NE Iran and Bacharden, as well as the diploid specimen from Bishkek, exhibited values mostly smaller than 310 μm^2. The distinction of diploid and tetraploid green toads from these populations is possible with an error rate of only about 3.3 %: among 36 diploid toads, only 1 individual displayed a value higher then 310 μm^2; among 85 tetraploids, 3 had a value lower than 310 μm^2. The values of the average erythrocyte area in the population Bol'shoi Balkhan were situated in the overlapping range between diploid and tetraploid toads values and are therefore difficult to use for identification. In this population, the two investigated animals were identified as tetraploid (erythrocyte areas 292 μm^2 and 322 μm^2; see tab. 2), but the occurrence of diploid or triploid specimens in the sample could not be fully excluded (see *Discussion*).

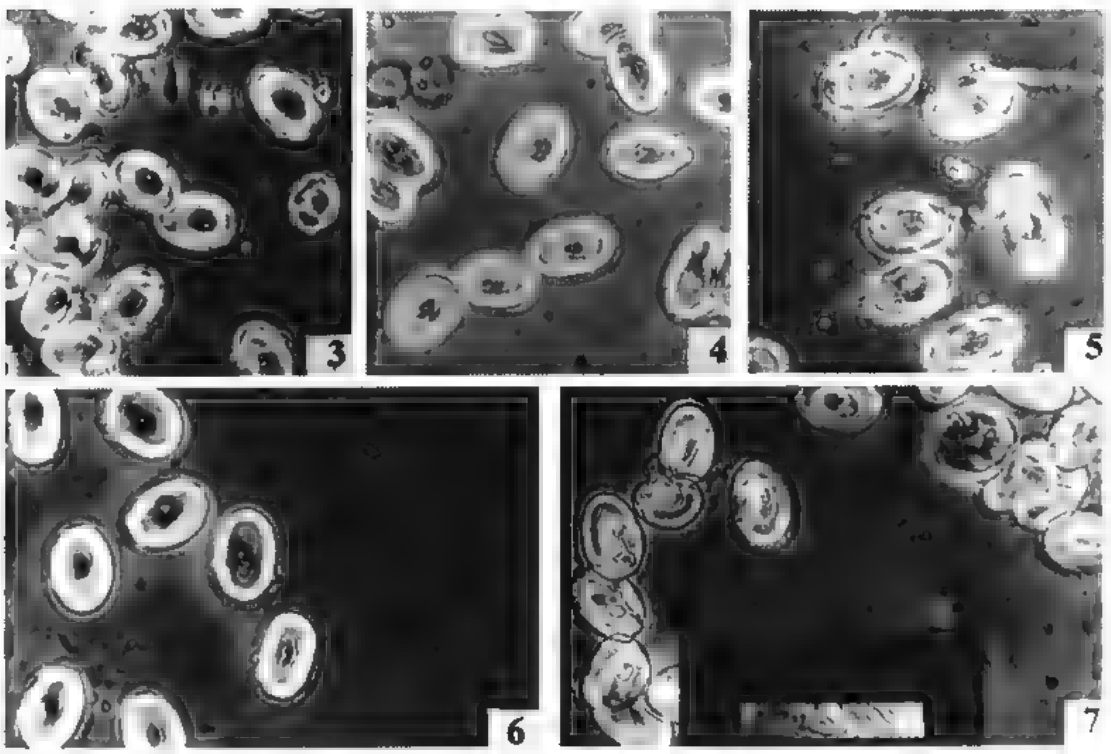

Fig. 3–7. Sections of blood smears from two diploid and tetraploid green toads. (3) Diploid individual from NE Iran; average value of erythrocyte projection area: 258.53 μm^2. (4) Diploid individual from Bishkek; 279.78 μm^2. (5) Tetraploid individual from Danata; 367.94 μm^2. (6) Tetraploid individual from Issyk-Kul'; 363.67 μm^2. (7) Tetraploid individual from Nuratau, 311.14 μm^2.

Taking the population Bol'shoi Balkhan into consideration, there is an overlapping range between 287 μm^2 (minimal average value of tetraploids; $n = 98$) and 318 μm^2 (maximal average value of diploids; $n = 36$). This range includes the values of eight diploids from the population Bami and of 21 tetraploids (10 from Bol'shoi Balkhan, $n = 13$; 6 from Nuratau, $n = 36$; 2 from Tashkent, $n = 6$; 2 from Chatkal, $n = 5$; 1 from Danata, $n = 17$). Therefore this method only allowed to classify unambiguously 77.8 % of diploid and 78.6 % of tetraploid green toads. Figures 3-4 show sections of blood smears of two diploid specimens from the populations NE Iran and Bishkek. Examples of tetraploid specimens from the populations Danata, Nuratau and Issyk-Kul' are to be seen in fig. 5-7.

Erythrocyte areas of the individuals

In all specimens investigated, a high variability of erythrocyte areas was found. Minima and maxima in a sample (one sample measured from each toad) were up to 25 % above or below the average value. However, despite the resulting fluctuation in standard deviation ($s = 25\text{-}35$ μm^2), we consider that the measurement of 30 or 50 erythrocyte areas gives a good representation of the erythrocytes of an individual.

Erythrocyte areas of the populations

When enough animals could be tested, the variation range was found to vary between populations: from 39 μm^2 between minimum and maximum value in Bol'shoi Balkhan (12 % of the average value; $n = 13$), through 48 μm^2 in NE Iran (18 % of the average value; $n = 9$), 60 μm^2 in Bami (21 % of the average value; $n = 23$), 63 μm^2 in Danata (18,5 % of the average value; $n = 17$), 72 μm^2 in Nuratau (21,5 % of the average value, $n = 36$), and up to 91 μm^2 in Issyk-Kul' (24,3 % of the average value; $n = 21$; see fig. 2). Variation covers a similar range in the diploid and tetraploid populations. Although in some cases the number of specimens is too low to be statistically meaningful, distinct differences of the average values between some populations can be noticed (see tab. 2). This is clear especially in the population Issyk-Kul' whose average value of 374 μm^2 is considerably higher than in all other tetraploid populations studied. To compare the average values of all populations, an analysis of variance was performed. The average value of each diploid population was significantly different from each tetraploid population (Multiple Range test/LSD test; $P < 0.05$; see tab. 3). Furthermore it was found that the average value of erythrocyte areas in the population Issyk-Kul' is significantly different from every other diploid and tetraploid population. While the tetraploid populations Danata, Nuratau, Tashkent and Chatkal seem to be quite homogeneous (but sample sizes in Tashkent and Chatkal are small), and exhibit no significant differences between each other, the population Bol'shoi Balkhan occupies, as expected, a special position. Its average value is different from those of the populations Danata, Nuratau and Chatkal (but not from Taskent), and also from each diploid population (NE Iran, Bacharden, Bami). The average value of the population Halle from Central Europe differs from each average value of the Middle Asian populations. With 231.89 μm^2, it is smaller than the minimal values of the Middle Asian populations (NE Iran: 264 48 μm^2; Bacharden: 243.79 μm^2).

Relationship between body size, age and erythrocyte size

A ratio of erythrocyte area to snout-urostyle length within a population was considered useful for testing a dependence of the parameter on age or body size respectively. Figure 8

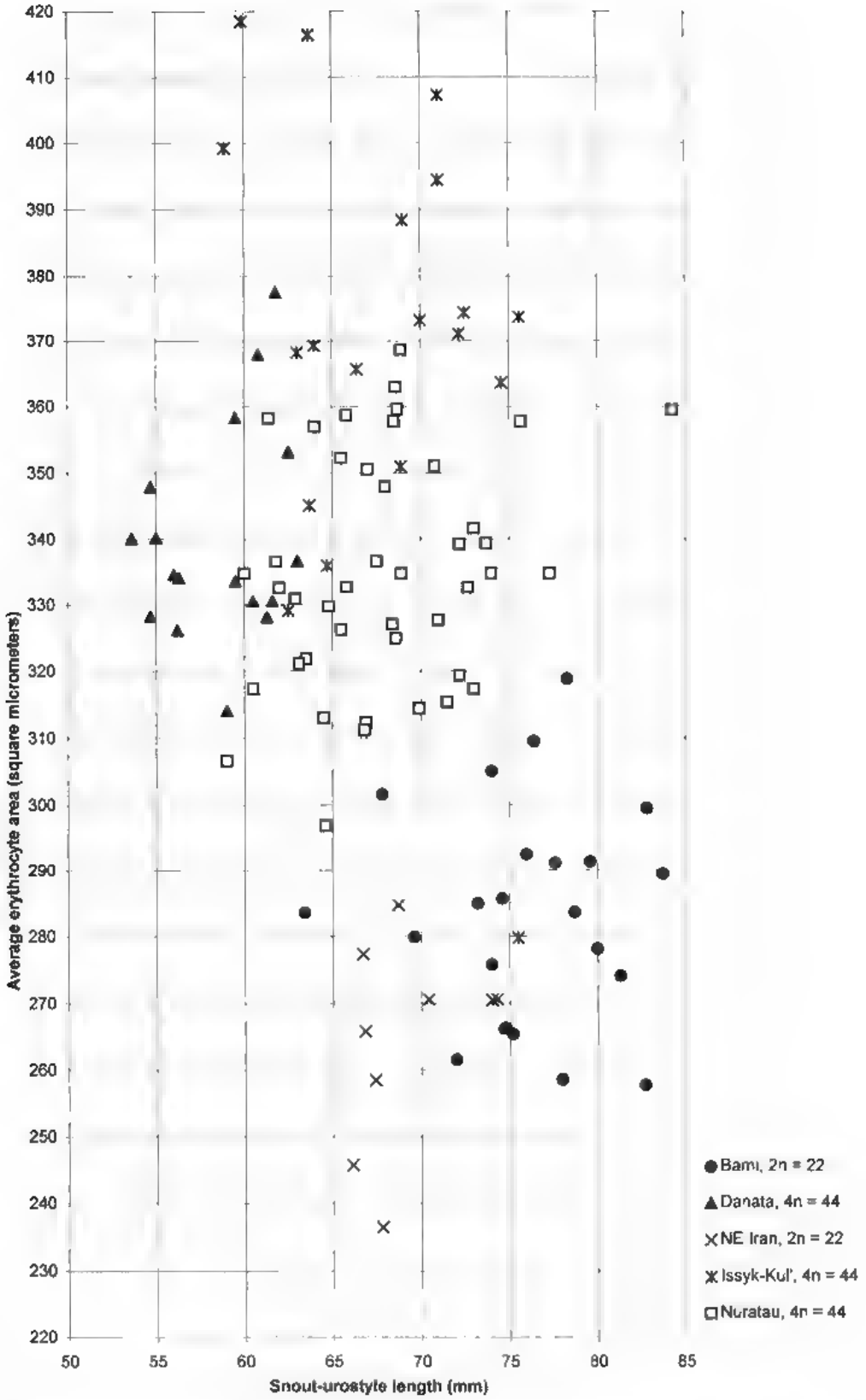

Fig. 8 Erythrocyte size and body size in two diploid and three tetraploid green toad populations.

shows this relation for two diploid and three tetraploid populations. Body size, although exhibiting high variation, showed no correlation with erythrocyte size. Therefore, body size does not affect the identification of ploidy levels of mature animals. However, the additional investigation of some tetraploid juveniles (snout-urostyle length 3 to 5 cm) from the population Issyk-Kul' showed values (327, 327 and 342 μm^2) which were at the lower border of the values within this population. Four juveniles (snout-urostyle length 4.5 to 5 cm) from the population Nuratau had similarly low values (290, 306, 316 and 317 μm^2); a juvenile from Danata (3.5 cm) had the second smallest value within this population.

Erythrocyte areas, sex and altitude of the habitat

There is no noticeable sexual dimorphism in this character: within the populations, the values of the (underrepresented) females were uniformly distributed among the values of males. No relationship was found between erythrocyte area and altitude (see tab. 2) of toad habitats.

Microdensitometrical measurements

Stored, air dried blood smears

The nuclei in air dried blood smears which were stored without freezing some weeks or months were not suitable for quantitative staining with the Feulgen reaction. The older the sample was, the less it could be stained.

Fresh fixed blood smears

On the contrary, fresh fixed blood smears exhibited very exact results. Figure 9a shows as an example the frequency distribution of the DNA amounts measured in 100 erythrocyte nuclei of a diploid (DNA index I) specimen from the population NE Iran. Figure 9b demonstrates the DNA amounts of 200 nuclei of a tetraploid (DNA index II) from the population Issyk-Kul'. Since the total DNA amount of a nucleus is measured, what could in principle result also from an aneuploid chromosome number, in such measurements the DNA amount of a diploid nucleus is named as DNA index 1 and relative values were analysed.

Discussion

Erythrocyte areas

Method

The measurement of erythrocyte areas yields useful results for distinguishing between diploid and tetraploid green toads. Combined with the hitherto presented morphological characters (Pisanets, 1978; Pisanets & Ščerbak, 1979; Kuzmin, 1995) or with multivariate analyses of morphometrical data (Stöck, 1997), the method offers new opportunities for the determination of Middle Asian green toads. In spite of this study including only one diploid

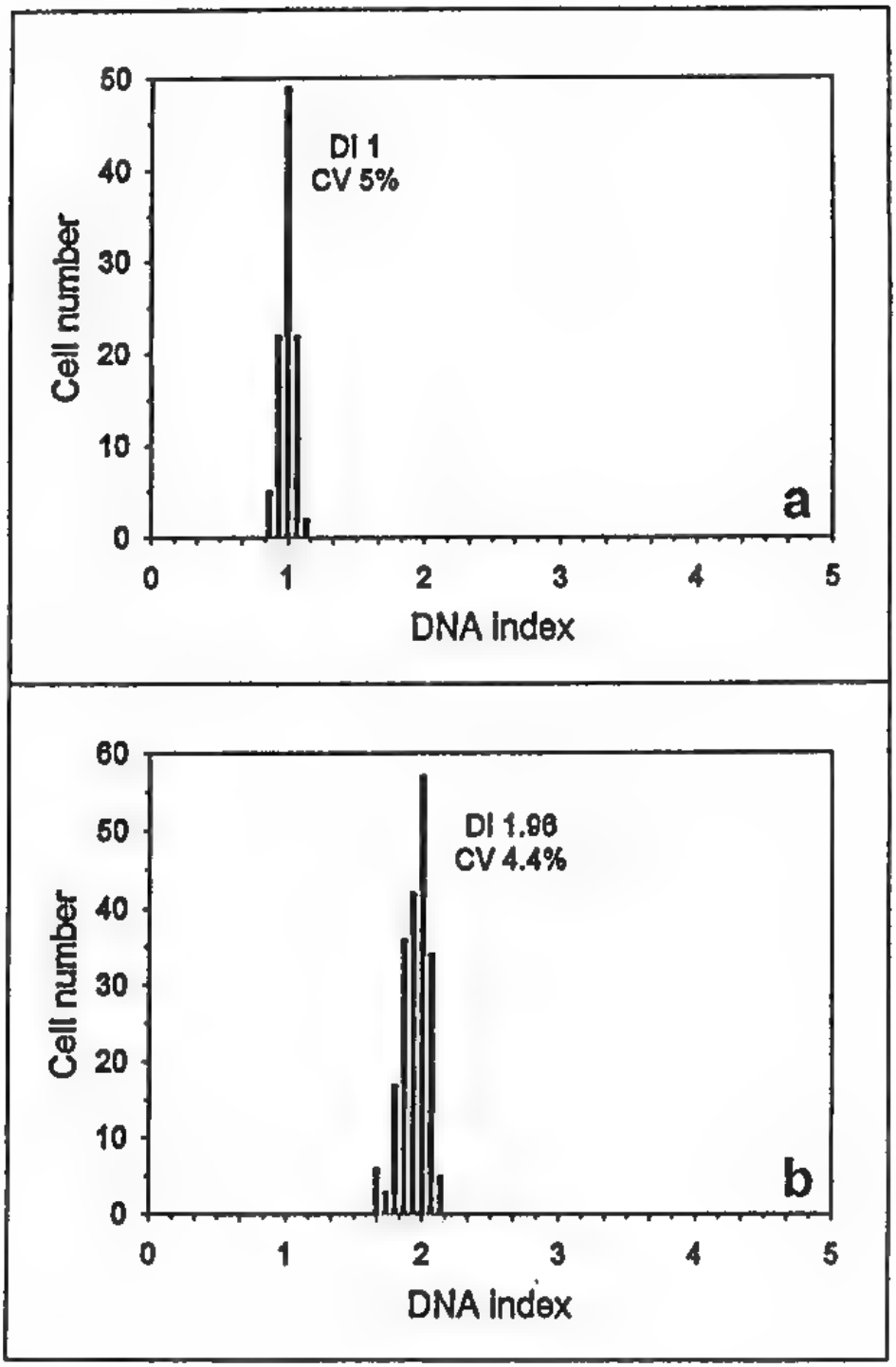

Fig 9 – (a) Frequency distribution of DNA amounts of 100 erythrocyte nuclei of a diploid green toad individual (DNA index DI 1) from population NE Iran. CV, coefficient of variation (%). (b) Frequency distribution of DNA amounts of 200 erythrocyte nuclei of a tetraploid green toad (DNA index DI 1.96) from population Issyk-Kul'. CV, coefficient of variation (%).

animal from the East of the range (Bishkek, Kyrgyzstan), with reference to BACHMANN et al. (1978) and to the DNA measurements (based on flow cytometry) by BORKIN et al. (1986), it can be postulated that the method can be applied also in this region, all the more that tetraploids in this region (Issyk-Kul') are characterised by especially large erythrocytes. The method can help to enlarge the knowledge about distribution of diploid and tetraploid green toads, which is desirable to clarify taxonomic problems (BORKIN et al., 1986).

However, this study has shown that there are populations (Bol'shoi Balkhan) whose values of erythrocyte areas are between most of the other diploid and tetraploid populations. In such cases, or if there are specimens with average values between about 285 and 320 μm^2, it is necessary to use methods such as karyological, cytophotometrical or microdensitometrical analyses (see below).

There were only few records of triploid hybrids in nature, in Danata (PISANETS, 1978; MEŽŽERIN & PISANETS, 1995*a-b*) and near Bishkek (KUZMIN 1995: 187, without reference to the method of ploidy determination), and only few references to "mixed populations", in Dushanbe (ROTH & RÁB, 1986) and in East Kazakhstan (GOLUBEV, 1990, but ploidy determination in this case seems doubtful). Consequently, we first supposed that there were only toads of one level of ploidy at each investigated locality. In none of the populations an inconsistency was found between ploidy of adults and ploidy of larvae using karyological or microdensitometrical methods (see tab. 2; STÖCK, 1995) Among adult animals, a few had average values of erythrocyte areas that were situated in the overlapping range between diploid and tetraploid toads values (marked * in tab. 2). However, all these individuals had the same ploidy as other adult toads and tadpoles of the population. So, it seems very improbable that there were any "mixed population" among those studied.

Variability of erythrocyte areas

In the present study, the number of red blood cells investigated per individual (30 or 50) seems to be large enough, in particular in comparison with literature (OLMO & MORESCALCHI, 1975: 15 cells per individual; GÜNTHER, 1977: 50 cells; KURAMOTO, 1981: more than 20 cells; AUSTIN & BOGART, 1982: more than 20 cells; POLLS PELAZ & GRAF, 1988: 10 cells; SCHRÖER, 1996: 15 cells) The standard deviations within the samples are of the same magnitude as those presented by KURAMOTO (1981) or POLLS PELAZ & GRAF (1988) for some other anuran species.

High variability within the cell sample from a specimen could be caused by the occurrence of immature erythrocytes (erythroblasts) in the peripheral blood of Amphibia (ZAPATA et al., 1982; WELSCH & STARCK, 1986), a presence that has been shown by KHALIL & ELFEKY (1986) to occur in green toads of the *Bufo viridis* complex. Such cells have a tendency to exhibit a circular shape. Among the cells randomly chosen for measurement, this cell type was included too. Apparently the spleen is the principal erythropoietic organ in temperate adult anurans (DUELLMAN & TRUEB, 1994: 180), but due to the activity of the bone marrow as an erythropoietic site during spring (DUELLMAN & TRUEB, 1994: 180), there could be seasonal shifts in the frequency of different erythrocyte sizes. TOKTOSUNOV (1984) noted an increased number of erythrocytes during the cool seasons (autumn, winter, spring) in diploid green toads from Chu valley near Bishkek (Kyrgyzstan). Because of the uniform period of investigations (breeding time) in our study, seasonal effects are thought to be quite constant (see GÜNTHER, 1977). Actually, about half of the individuals from the population Issyk-Kul' were

investigated in autumn, but the range of the values did not diverge from the values in animals investigated in spring. Exclusively some maxima were only detected in spring.

Although the variation range of the erythrocyte areas in all polyploid individuals in the present study is larger than in all diploids (a result similar to GÜNTHER's, 1977, findings in triploid *Rana* kl. *esculenta*, but in contradiction with those of POLLS PELAZ & GRAF, 1988, in the same klepton), a relationship between this phenomenon and polyploidy cannot be clarified. In triploid representatives of the *Ambystoma jeffersonianum* complex, AUSTIN & BOGART (1982) found about the same variability of the erythrocyte areas as in diploid

The erythrocyte measurements by HEMMER et al. (1978) in toads of unknown ploidy from the environs of Dushanbe (Tadzhikistan) could not be compared with the data presented here. On the one hand, these authors measured length and breadth of the ellipsoid and then calculated the ellipse area, and on the other hand they made a Pappenheim staining which might result in a shrivelling of the cells. That might be the reason why their values are smaller than those presented here. Interestingly however, HEMMER et al. (1978) registered high variability in the whole studied sample of toads (i.e. in diploid and tetraploid individuals), a result in agreement with ours. The differences between populations are striking too in toads of the same level of ploidy. In Israel and Greece (areas quite small compared to the giant region taken into consideration here), an enormously high genetic variability was found in diploid *Bufo viridis* (DESSAUER et al., 1975: "the highest yet reported for any vertebrate"; KARAKOUSIS & KYRIAKOPOULOU-SKLAVOUNOU, 1995). Therefore it is not surprising to find a great phenotypic variety in tetraploid green toads, particularly because during evolution, polyploidy was presumably associated with long periods of isolation between many different populations in high mountains (see ROTH, 1986). The phenomenon seems to be confirmed by measurements of DNA contents in erythrocytes (BORKIN et al., 1986): these authors suggested the probable existence of three groups of diploid and two groups of tetraploid toads in Middle Asia. Considering the positive correlation mentioned above, a direct relationship between DNA amount and cell volume (and erythrocyte area) is probable.

Other polyploid amphibians and their differences in erythrocyte areas

Contrary to the situation in the *Ambystoma jeffersonianum* complex, in which diploid and triploid individuals can be distinguished without errors using the very large erythrocytes of the Urodela (AUSTIN & BOGART, 1982), GÜNTHER (1990: 172) and SCHRÖER (1996) mentioned that in some populations of water frogs it is problematical to distinguish between diploid and triploid *Rana* kl. *esculenta* as reported by GÜNTHER (1977). Artificially produced triploid individuals of *Xenopus laevis* exhibited a significant difference between the average values but an overlapping of the absolute values of the largest cell axis compared with diploid forms (GEORGE & LENNARTZ, 1980). MAHONY & ROBINSON (1980) found clear differences in the average values of the erythrocyte areas in the tetraploid Australian leptodactylids *Neobatrachus sudelli* and *N. sutor* compared with those of diploid *N. pictus*; however, this work contains no information on variation range or intrapopular variability. BOGART & WASSERMAN (1972), using two microphotographs, demonstrated the different sizes of erythrocytes in the diploid/tetraploid species pair *Hyla chrysoscelis*/*H. versicolor*, which was confirmed by RALIN (1977) and CASH & BOGART (1978); an extensive study of erythrocyte size in this complex has still not been published.

Relation to age, body size and altitude

The slight increasing of erythrocyte size during ontogenesis till adulthood, as it seems to develop in (diploid and) tetraploid green toads, corresponds to the findings in diploid and triploid *Rana* kl *esculenta* (POLLS PELAZ & GRAF, 1988) whose erythrocyte areas enlarge with increasing body size after metamorphosis. In *Bufo melanostictus*, CHURCH (1961; cited by GÜNTHER, 1977) and BANERJEE (1983, 1988) observed an enlargement of erythrocyte areas with increased body size. In *Bufo spinulosus*, RUIZ et al. (1989) noticed a reduction of erythrocyte area and body size with increasing altitude, whereas erythrocyte number per volume unit was increased (the authors compared individuals from 200 to 2700 m with individuals living above 3200 m) The authors considered this phenomenon an adaptation to the reduced oxygen content in the air of the high mountains. Our investigations do not show any relationship between erythrocyte size and altitude of the habitat. The investigated green toad population living at the highest altitude (Issyk-Kul', 1670 m) displays the highest average value of erythrocyte area. In tetraploid green toads from this area, TOKTOSUNOV (1984) found a higher haemoglobin content (147 g/l) than in diploid lowland populations (112 g/l) and considered this an adaptation of the tetraploids to their mountainous life. It is still unknown whether tetraploid green toads which inhabit high mountains regions above 3500 m (MAZIK et al., 1976; ROTH, 1986; KUZMIN et al., 1988; TOJMASTOV, 1989), and which exhibit in higher altitudes a reduced body size (TOJMASTOV, 1989), have a reduced erythrocyte area too.

MICRODENSITOMETRICAL MEASUREMENTS

DNA amounts

The DNA amount in cells of the tetraploid green toads tested is about twice the content in nuclei of diploid toads. This result is in agreement with those of BORKIN et al. (1986), who found in tetraploids an amount slightly lower than the double of diploids.

Method

This method seems to be the only procedure usable without killing or considerably damaging the animals to determine unequivocally their ploidy. The Feulgen process cited above (HORNER & MACGREGOR, 1983) is suitable for erythrocytes in fresh blood smears from Middle Asian green toads. Up to now it is unknown how long blood smears could be stored if they were fixed as mentioned before staining them with the Feulgen reaction. A test is in preparation. If it turns possible to store blood smears some time without loss in quantitative Feulgen staining, this method could become a key to collect data about distribution of different ploidy types in Middle Asian green toads without killing the specimens. Such a method would be most useful in faunistics and other field investigations.

RÉSUMÉ

Les tailles des érythrocytes de populations de crapauds verts (complexe de *Bufo viridis*) diploïdes et tétraploïdes de l'Asie centrale (Iran, Turkménistan, Ouzbékistan et Kirghizstan) sont comparées en mesurant leur surfaces de projection à partir de frottis sanguins. Les valeurs moyennes de ces surfaces sont significativement différentes entre les deux types de crapauds. Dans quelques populations les valeurs moyennes sont intermédiaires entre celles des animaux diploïdes et tetraploïdes. La variabilité des surfaces des érythrocytes par rapport à l'âge, la taille et l'altitude, est discutée à la lumière de la littérature sur les crapauds verts et les autres anoures polyploïdes. La coloration des noyaux des érythrocytes par la réaction de Feulgen et la détermination microdensitométrique du contenu de l'ADN sont décrites. Cette méthode est préconisée pour obtenir des résultats clairs sans sacrifier les animaux.

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